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TITLE: The Role of mDia1 in the Aberrant Innate Immune Signaling in del(5q) Myelodysplastic Syndromes

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14. ABSTRACT

Myelodysplastic syndromes (MDS) are a group of diseases affecting bone marrow and blood with an increased risk of developing acute leukemia. Many genomic abnormalities are associated with MDS with deletion of chromosome 5q being the most common. Our recently published work demonstrated that loss of mDia1, a protein with its encoding genes located at chromosome 5, led to the activation of the innate immune response through an aberrant overexpression of CD14 on granulocytes, which accelerate the development of MDS in mDia1 deficient mice. Based on this study, we hypothesis that CD14 induced abnormal immune response is critical in the pathogenesis of MDS. In the past grant reporting cycle, we have successfully demonstrated the significance of in vivo damage associated molecular patterns (DAMPs) in the pathogenesis of MDS. Significant achievements were also obtained in other major goals. We demonstrated the efficacies of CD14, AP1, and SP1 inhibitors in the downregulation of CD14 in granulocytes in vitro and in vivo. CD14 and mDia1 double knockout are also generated and we expect to have data in a few months. There are no major changes of the experimental designs.

15. SUBJECT TERMS

Myelodysplastic syndromes, del(5q), mDia1, CD14, innate immune signaling, TLR4, DAMPs, PAMPs, miR-146a, CD11b, neutropenia, endocytosis, inhibitors, mouse model

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Table of Contents

Page

INTRODUCTION

The overall goal of this project is to determine the role of mDia1 in the innate immune signaling and the pathogenesis of del(5q) MDS. Deletion of chromosome 5 long arm (del(5q)) is the most common cytogenetic abnormality in patients with myelodysplastic syndromes (MDS). We discovered in 2014 that CD14 was aberrantly overexpressed on granulocytes of mice with loss of mDia1, whose encoding gene is located at 5q31. This led to a hypersensitive innate immune response to the stimuli of lipopolysaccharide (LPS) in mDia1 heterozygous and knockout mice. Importantly, chronic stimulation of these mice with LPS accelerated the development of MDS that normally occurs in aged mDia1 deficient mice. CD14 was also found overexpressed on granulocytes of patients with del(5q) MDS. Based on these published results, we hypothesize that mDia1 deficiency induced aberrant innate immune signaling, through CD14 and its co-receptor TLR4, is critical for the pathogenesis of del(5q) MDS. To test this hypothesis, we proposed three specific aims. In aim 1, we will determine how damage associated molecular patterns (DAMPs), as the in vivo ligands for CD14/TLR4, are involved in the hypersensitive innate immune response and development of MDS in mDia1 deficient mice. In aim 2, we will determine how CD14 is involved in the development of MDS in mDia1 deficient mice using CD14/TLR4 inhibitors and a novel CD14/mDia1 double knockout mouse model. Aim 3 will focus on the mechanism of CD14 upregulation with loss of mDia1. The possible role of transcriptional repression of CD14 in rescuing MDS in mDia1 deficient mice will also be determined. Successful accomplishment of our proposed project is important for the understanding of the pathogenesis of aberrant innate immune responses in del(5q) MDS. The new CD14/mDia1 double knockout mouse model and novel concept of using pharmacologic inhibitors to target CD14/TLR4 would also greatly help the development of novel immunotherapeutic management for MDS.

KEYWORDS

Myelodysplastic syndromes, del(5q), mDia1, CD14, innate immune signaling, TLR4, DAMPs, PAMPs, miR-146a, CD11b, neutropenia, endocytosis, inhibitors, mouse model.

ACCOMPLISHMENTS

The major goals of the project:

The major goals of the project are listed below. The completion dates or the percentage of completion are noted.

- Major goal 1: Determine the role of DAMPs in the hypersensitized innate immune response in mDia1 deficient granulocytes in vitro.
    - Completed in August 2016.
- Major goal 2: Injection of DAMPs to mDia1 heterozygous and knockout mice to determine whether they can induce phenotypes mimicking del(5q) MDS.
    - Completed in February 2016
- Major goal 3: Determine the effect of CD14 inhibitors on the response of mDia1 deficient mice treated with LPS or DAMPs.
    - Percentage of completion: 70%
- Major goal 4: Determine whether knockout of CD14 would rescue the hypersensitized innate immune responses in mDia1 deficient mice.
    - Percentage of completion: 50%
- Major goal 5: Determine the transcription factors that are responsible for CD14 upregulation during granulocytic differentiation in vitro in mDia1 deficient Gr1/Mac1 double positive cells.
    - Completed in July 2017
- Major goal 6: Determine whether mDia1 directly interacts with AP1 or SP1 to influence their transcriptional regulation of CD14.
    - Percentage of completion: 60%
- Major goal 7: test whether inhibition of CD14 transcription pharmacologically would abolish CD14 overexpression and myelodysplasia.
    - Percentage of completion: 70%

Accomplished work:

The accomplished work of each major goal is described below.

<u>Major goal 1: Determine the role of DAMPs in the hypersensitized innate immune response in mDia1 deficient granulocytes in vitro.</u>

This goal is completed. We have submitted our manuscript to journal ***Leukemia*** and received favorable comments. We are working on the revision of manuscript and will resubmit to *Leukemia* soon. **The manuscript is attached in the appendices**. In this manuscript, we generated a mDia1 and miR-146a double knockout mouse model which exhibited more severe MDS phenotypes than mDia1 or miR-146a single knockout mice. The rationale of using miR-146a knockout mouse model in this study is that we can

better evaluate the role of mDia1 in a more pathologically relevant background since miR-146a is also located on chromosome 5q. In addition, miR-146a is also involved in the innate immune signaling. Loss of miR-146a in mice causes a similar hyperactivated innate immune response similar to that in mDia1 knockout mice.

The mDia1/miR-146a double knockout (DKO) mice showed age-related severe anemia and thrombocytopenia compared to normal control and single knockout mice. Given the significance of the bone marrow microenvironment in the development of anemia, we examined the levels of proinflammatory cytokines in old mDia1/miR-146a double knockout mice and littermate controls. As expected, serum levels of both IL-6 and TNFα were significantly increased in old DKO mice compared to age-matched wild type and single knockout controls (**Figure 1**). The ageing bone marrow microenvironment is composed of increasing amounts of DAMPs, which are potent inducers of proinflammatory cytokines production. We previously demonstrated that mDia1 deficient Gr1/Mac1 positive granulocytes over-produced TNFα and IL-6 when treated with LPS. A similar phenotype was also observed in miR-146a knockout mice. To analyze whether treatment of DAMPs could also induce the over-production of proinflammatory cytokines in old double knockout mice in vitro, we purified Gr1 and Mac1 double positive granulocytes from the bone marrow and spleen of young DKO mice and littermate controls. These cells were treated with DAMPs prepared through repetitive freeze thaw cycles of wild-type bone marrow cells. Similar to LPS, treatment with DAMPs induced over-production of TNF-α and IL-6 in the bone marrow- and spleen-derived myeloid cells from each group of mice, with levels particularly high in double knockout cells (**Figure 2**). These results indicate that bone marrow microenvironment-mediated ineffective erythropoiesis in double knockout mice could be related to the over-secretion of proinflammatory cytokines.

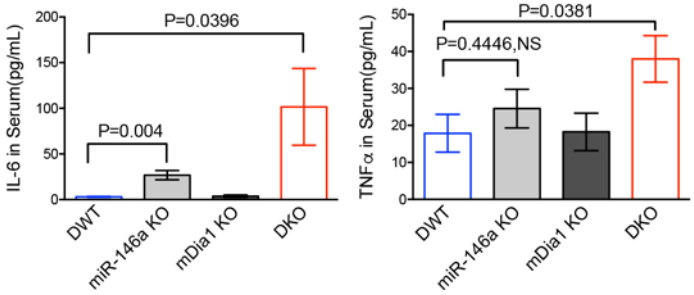


Figure 1

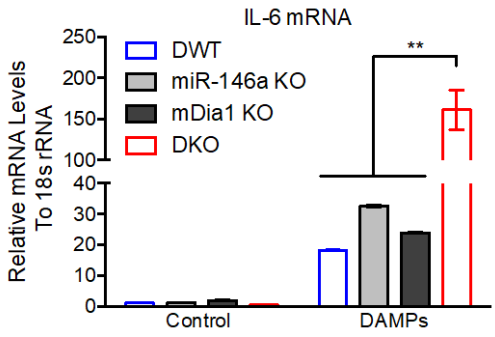


Figure 2

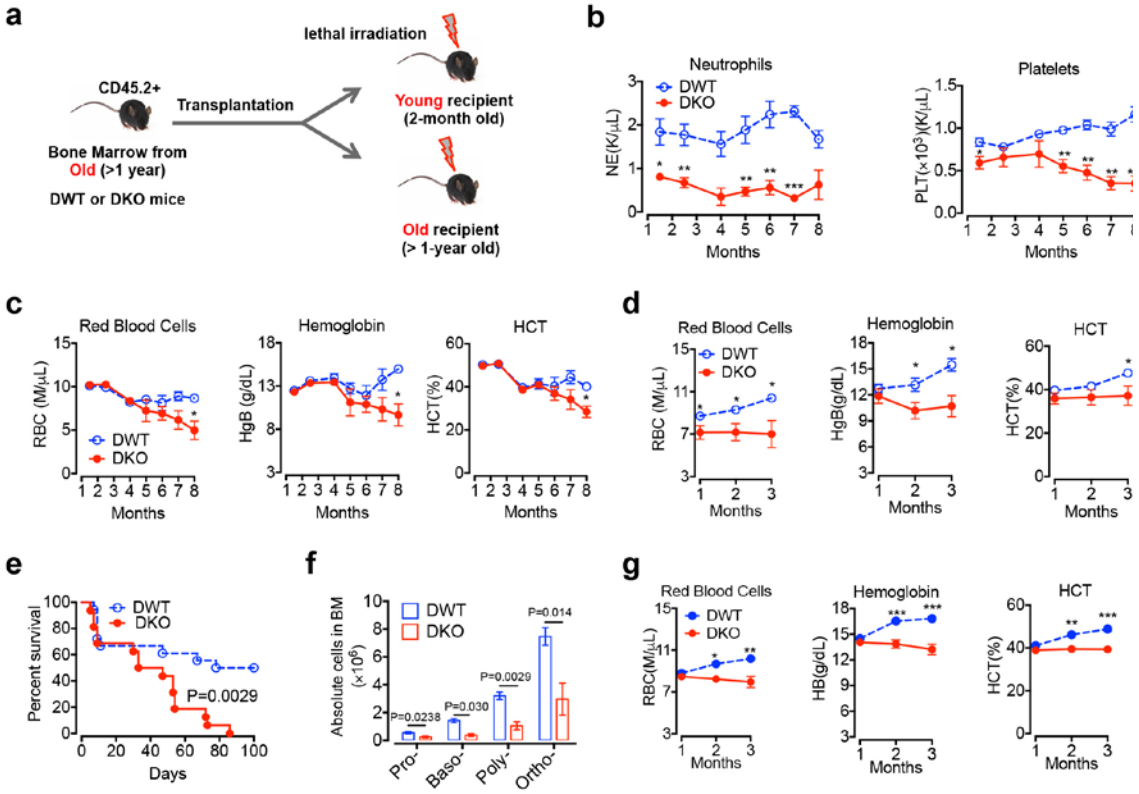
Major goal 2: Injection of DAMPs to mDia1 heterozygous and knockout mice to determine whether they can induce phenotypes mimicking del(5q) MDS.

We injected DAMPs in mDia1 heterozygous and knockout mice. The same injection experiment was also performed in mDia1/miR-146a double knockout mice. We tried several different forms of DAMPs for the in vivo injection. In the in vitro experiments described in major goal 1, we used bone marrow cell lysate from repeated freeze-thaw cycle as DAMPs, which include a mixture of various components including proteins and nucleate acids. In vitro experiments as described above showed potent abilities of these DAMPs to induce aberrant proinflammatory cytokine production in neutrophils. However, in vivo injection of these mixture of DAMPs did not give promising results, which could be due to the impure nature of the DAMPs. We also performed injection with known DAMPs including HMGB1, which did not show good results either.

Instead of injection of DAMPs, we changed our experimental strategy and transplanted mDia1 or DKO bone marrow cells into aged recipient mice. In this way, the transplanted bone marrow cells encounter the aging bone marrow microenvironment that is enriched with DAMPs. In these experiments, bone marrow mononuclear cells from aged (> 1-year old) DKO mice or age matched double wild type (DWT) littermate controls were transplanted into either young (2-month old) or aged (> 1-year old) recipient mice (**Figure 3a**). Like the non-transplanted aged DKO mice, these transplanted mice exhibited neutropenia early post-transplantation due to the cell-intrinsic effect of mDia1 deficiency, as well as worsening thrombocytopenia (**Figure 3b**). The young recipient mice showed no significant differences in red cell indices between DKO and DWT transplanted mice until 6-month post-transplantation when RBC count, hemoglobin, and hematocrit began declining (**Figure 3c**). The age when anemia manifested in these transplanted mice was similar to that of the regular DKO mice, implicating an age-related microenvironment in the development of anemia.

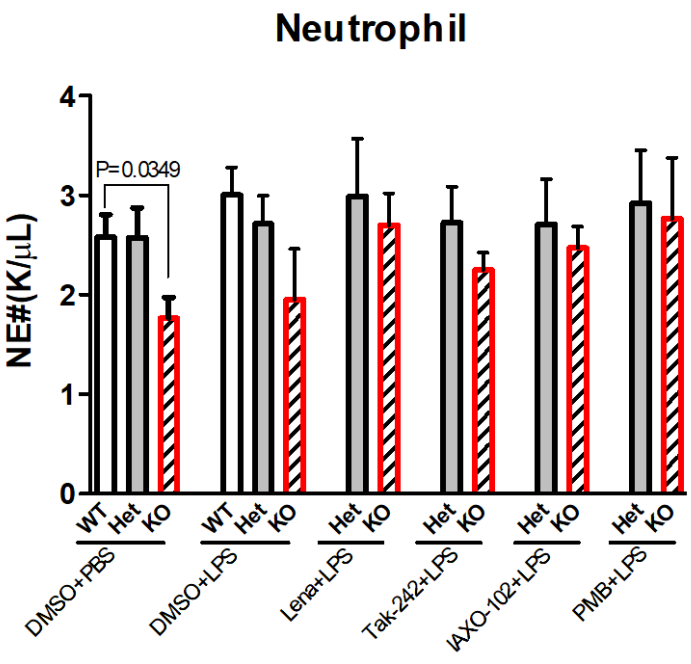
Old (> 1-year of age) recipient mice transplanted with old DWT or DKO bone marrow cells showed initial anemia due to their age. Those transplanted with DWT bone marrow cells gradually recovered from transplantation stress. However, the old recipient mice transplanted with old DKO bone marrow cells exhibited persistent anemia starting from one month post-transplantation (**Figure 3d**). The old recipient mice transplanted with old DKO bone marrow cells also exhibited rapid lethality compared to the old DWT bone marrow transplanted controls (**Figure 3e**). When we analyzed the bone marrow of the old recipient mice from both groups of the transplantation experiments, we found ineffective erythropoiesis at different stages of red cell development similar to the non-transplanted old DKO mice (**Figure 3f**). Furthermore, when we transplanted young DKO bone marrow cells into old recipient mice (> 1-year of age), the recipient mice failed to recover from anemia compared to DWT transplanted ones (**Figure 3g**). Taken together, these results indicate that the age-related bone marrow microenvironment plays an important role in the development of ineffective erythropoiesis and anemia.

Figure 3



Major goal 3: Determine the effect of CD14 inhibitors on the response of mDia1 deficient mice treated with LPS or DAMPs.

Figure 4



We have performed preliminary studies on this major goal. Specifically, mDia1 wide type (WT), heterozygous (Het) and knockout (KO) mice were intraperitoneally injected with 1 mg/kg Lipopolysaccharide (LPS) simultaneously with various inhibitor as indicated in the Figure every 2 weeks for 5 consecutive months. Lena indicates lenalidomide (10mg/kg), which is commonly used to treat del(5q) MDS. Tak-242 (3mg/kg) is a commercially available NF-kB inhibitor.

IAXO-102 (1mg/kg) is a commercially available CD14 inhibitor. PMB is Polymyxin B (2000U/kg), antibiotics against Gram-negative bacteria. Complete blood counts were performed after the last injection and neutrophil counts were shown in the **Figure 4**. As shown, mDia1 KO mice showed prominent neutropenia. LPS treatment increased neutrophil counts in wild type mice, which was not present in mDia1 Het and KO mice. As we expected, lenalidomide, NF-kB inhibitor, CD14 inhibitor, and antibiotics all rescued neutrophil count in mDia1 knockout mice. These data suggest that CD14 inhibitor plays a similar role as other commonly used compounds for treating cytopenia, thus providing basis for the possible future development of CD14 inhibitors to treat MDS.

We are also in the process of testing the effect of CD14 inhibitors in rescuing the phenotypes in mDia1/miR-146a DKO mice. We have started injecting IAXO-102 chronically at a low dose (0.2mg/kg) to DKO mice and their littermate DWT control mice. These mice will be followed for the next 6 months for their development of anemia, neutropenia, and thrombocytopenia. The bone marrow flow cytometric and morphologic changes will also be analyzed. We expect that CD14 inhibitor will at least delay the progress of MDS phenotypes in DKO mice.

Major goal 4: Determine whether knockout of CD14 would rescue the hypersensitized innate immune responses in mDia1 deficient mice.

We have purchased CD14 knockout mice from Jackson laboratory. These mice have been crossed with mDia1 knockout mice. We have recently obtained a few CD14 and mDia1 double knockout mice. We are breeding more mice to have enough to be statistically significant. These mice will be followed for their development of neutropenia. The bone marrow flow cytometric and morphologic changes will also be analyzed. We expect that loss of CD14 will ameliorate the phenotypes in mDia1 knockout mice, when they are challenged with LPS injection.

We are also crossing CD14 knockout mice with mDia1/miR-146a DKO mice. Similarly, once we obtain CD14, mDia1, and miR-146a triple knockout mice, we will follow these mice for their development of anemia, neutropenia, and thrombocytopenia. The bone marrow flow cytometric and morphologic changes will also be analyzed. We expect that loss of CD14 will at least delay the progress of MDS phenotypes in DKO mice. Successful accomplishment of this goal will provide genetic evidence that CD14 is critical for the pathogenesis of MDS in DKO mice.

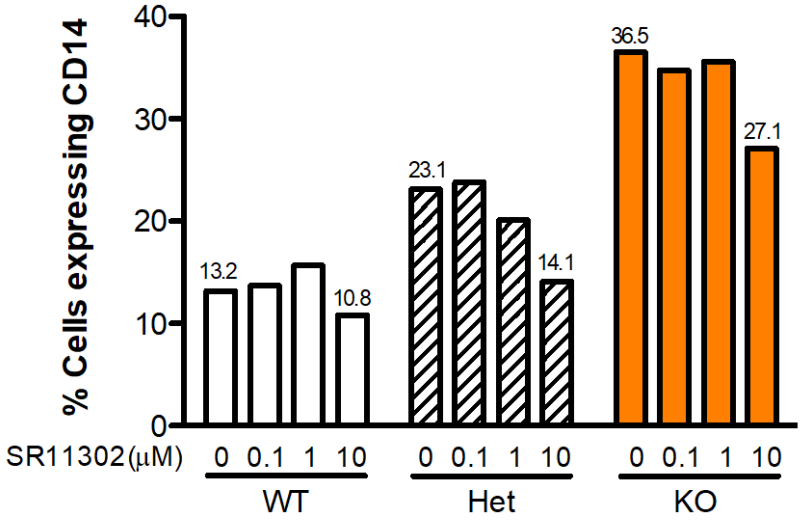
Major goal 5: Determine the transcription factors that are responsible for CD14 upregulation during granulocytic differentiation in vitro in mDia1 deficient Gr1/Mac1 double positive cells.

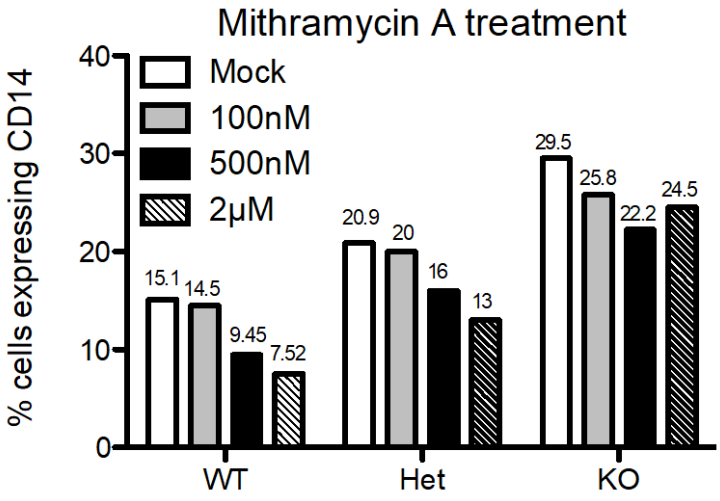
We have performed preliminary experiments using inhibitors of AP1 and SP1. As shown below in **Figure 5**, flow cytometry of cell surface CD14 levels on lineage negative cells cultured in vitro were performed. Specifically, we purified lineage negative bone marrow progenitor cells from mDia1 wild type (WT), heterozygous (Het), and knockout (KO)

mice. The cells were cultured for 3 days in GM-CSF medium with indicated amount of SR11302 (AP1 inhibitor) or Mithramycin A (SP1 inhibitor). The cells were harvested on day 3 and followed by cell surface CD14 analysis by flow cytometry. The data below show that both AP1 and SP1 inhibitor affected CD14 levels in mDia1 heterozygous and knockout bone marrow lineage negative cells differentiating to myeloid cells. These experiments establish the basis for the next step mechanistic study.

We also obtained similar results when AP1 or SP1 were knocked down by shRNA, which downregulated CD14 expression. As shown in **Figure 5**, inhibition of AP1 or SP1 does not completely abrogate the level of CD14. We further discovered that mDia1 also regulates CD14 at the posttranslational level. We found that loss of mDia1 influences the endocytosis of CD14 that controls its surface expression. Therefore, loss of mDia1 led to decreased CD14 endocytosis resulting in more CD14 on the cell surface.

Figure 5





We also found similar effect of loss of mDia1 in several other cell surface proteins including CD11b. In a recent published report in *Blood Advances*, we showed that mDia1 knockout mice show cell-autonomously increased CD11b expression on neutrophils in the peripheral blood and bone marrow. The level of CD11b was also higher in patients with del(5q) MDS compared to normal individuals. Mechanistically, loss of mDia1 significantly attenuated the endocytosis of CD11b on neutrophils, which led to an increased number of neutrophils in mDia1 knockout mice adhering to the blood vessels. Administration of CD11b antibody to mDia1 knockout mice reduced the adhesion of neutrophils to the vessels and rescued neutropenia. Our study reveals the role of mDia1 deficiency in the up-regulation of CD11b on neutrophil leading to their increased binding to the blood vessels. These results may provide important clues for the pathogenesis of neutropenia in del(5q) MDS.

Major goal 6: Determine whether mDia1 directly interacts with AP1 or SP1 to influence their transcriptional regulation of CD14.

We performed co-immunoprecipitation experiments using HET293T cells transfected with AP1 or SP1, together with mDia1. In the previous report, we indicated that there were some technical problems with the antibodies to AP1 and SP1. Strong background bands were detected. We tried several different antibodies but the results were not promising. Therefore we used a Flag-tagged mDia1 protein and HA-tagged AP1 and SP1 to perform the co-immunoprecipitation assay. The experiments are ongoing and we expect to obtain the results in a few months.

Major goal 7: test whether inhibition of CD14 transcription pharmacologically would abolish CD14 overexpression and myelodysplasia.

As demonstrated by Figure 5 in major goal 5, inhibition of AP1 or SP1 reduced CD14 expression level in mature myeloid cells. These data demonstrate that inhibition of CD14 transcription pharmacologically using AP1 or SP1 inhibitor will abolish CD14 overexpression in vitro.

We have started injecting AP1 or SP1 inhibitors in mDia1 heterozygous and knockout mice to determine whether CD14 levels will reduce in granulocytes. These are chronic injection and we have started the injection 2 months ago. We plan to inject the mice for 4 more months until they are 6-7 months old and develop MDS-like symptoms. As previous experiments, we will monitor these mice for their complete blood count, blood cell morphology, and flow cytometry for the development of MDS. These mice will also be sacrificed at the end of experiments to determine the bone marrow changes.

Opportunities for training and professional development:

During the past year of the funding period, my laboratory obtained additional funding supports internally and externally. I was also promoted as an Associate Professor of Pathology. Thanks to my career development committee members Drs. John Crispino and

William Muller! In the past years, they have been extremely helpful to provide me guidance scientifically and in professional development.

We will also have a monthly joint hematopoiesis group meeting with members from the laboratories of Drs. John Crispino and Elizabeth Eklund in the Division of Hematology/Oncology at Northwestern University. The finished part of the project has been selected for presentation at the annual American Society for Hematology (ASH) meeting. The finished part of the project also received favorable review from *Leukemia*. We expect to publish it in a few months. Work directly relevant to this work also was published in *Blood Advances* early in this year.

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

- Major goal 1: Accomplished
- Major goal 2: Accomplished
- Major goal 3: We will perform in vivo chronic injection of CD14 inhibitor and determine the role of CD14 inhibition on amelioration of MDS phenotypes in mDia1 knockout mice.
- Major goal 4: We have obtained a few CD14/mDia1 double knockout mice. More mice will be available soon. We will perform in vivo assays of these mice.
- Major goal 5: Accomplished
- Major goal 6: We will perform co-immunoprecipitation assays using tagged proteins.
- Major goal 7: We plan to perform in vivo injection of AP1 or SP1 inhibitors in mDia1 heterozygous and knockout mice to determine whether CD14 levels will reduce in granulocytes. We will also determine whether the inhibitors will abolish myelodysplasia after chronic injection. These experiments have started and ongoing.

IMPACT

- What was the impact on the development of the principal discipline(s) of the project?

  Our findings demonstrate that ageing related bone marrow changes can affect the development of myelodysplastic syndromes (MDS). It also has impact in the military field in which military personnel with increased exposure to external stimulations of their innate immune signals may have increased risk of developing MDS.

- What was the impact on other disciplines?

  Nothing to Report

- What was the impact on technology transfer?

  Nothing to Report

- What was the impact on society beyond science and technology?

  Nothing to Report

CHANGES/PROBLEMS

Nothing to Report

PRODUCTS

Nothing to Report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Peng Ji
Project Role:	PI
Nearest Person Month Worked:	4 (No Change from previous submission)

Name:	Yang Mei
Project Role:	Post-doctoral Fellow
Nearest Person Month Worked:	8 (No Change from previous submission)

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? Yes, please see below

Dr. Peng Ji
ACTIVE
Department of Defense W81XWH-15-1-0335 9/15/15 - 9/14/18 3.6 Calendar months
Career Development Award $120,000 directs/year
Title:The role of mDia1 in the aberrant innate immune signaling in del(5q) myelodysplastic syndromes
Aims of the project: to study the role of mDia1 in the innate immune response and how loss of mDia1 contributes to the pathogenesis of myelodysplastic syndromes.
Role: Principal Investigator
*Award that we're submitting the progress report for

R01DK102718 (Ji) 4/10/15 - 1/31/20 1.86 Calendar months
NIH/NIDDK $256,051 directs: year 3
Title: The roles of pleckstrin-2 as a functional node in erythropoiesis
Aims of the project: To determine the role of pleckstrin-2 in connecting erythropoietin and Rac GTPase signals in the regulation of erythropoiesis.
Role: Principal Investigator
Changes since last reporting period: No Change

Dixon Translational Research Grants Initiative 1/1/17 – 12/31/17 0.12 Calendar months
Northwestern Memorial Hospital $50,000 directs/year
Title: Targeting Plek2 as a Novel Approach for the Treatment of Myeloproliferative Neoplasms
Aims of the project: 1) To determine the effects of Plek2 inhibitors in ameliorating myeloproliferative phenotypes in a $JAK2^{V617F}$ knockin mouse model. 2) To determine the effects of Plek2 inhibitors in treating MPN mouse models induced by MPL or CALR mutations. 3) To determine the inhibitory effects of Plek2 inhibitors on CD34+ hematopoietic progenitor cells from MPN patients
Role: Principal Investigator
Changes since last reporting period: Awarded

Scholar Award #1348-18 7/1/17 – 6/30/22 4.02 Calendar months
Leukemia & Lymphoma Society $104,762 directs/year

Title: The role of Plek2 in the pathogenesis of myeloproliferative neoplasms
Aims of the project: 1) To determine the roles of Plek2 in the pathogenesis of $JAK2^{V617F}$-induced MPN. 2) To determine the mechanisms of Plek2 in mediating the activated JAK2 signaling. 3) To determine the roles of Plek2 in MPNs induced by CALR and MPL mutations
Role: Principal Investigator
Changes since last reporting period: Awarded

The following grant has ended:
Chicago Biomedical Consortium Catalyst Grant 3/1/15 - 2/28/17 0.24 Calendar months
Nuclear opening and histone release in mammalian terminal erythropoiesis
Role: Principal Investigator. $60,000 directs/year
Aims of the project: To determine the role of caspase-3 in nuclear opening formation and histone release in both human and mouse models of terminal erythropoiesis.

Crispino, John D.
Role on reporting project: Mentor

Active
R01HL112792 (Crispino) 08/01/13 – 04/30/21 1.80 Calendar
NIH/NHLBI $325,159
Aberrant Megakaryopoiesis in the Myeloproliferative Neoplasms
The aims of this proposal are to 1) Discover the mechanisms by which activated JAK/STAT signaling leads to downregulation of GATA1 and impaired megakaryopoiesis in PMF, and 2) Determine the role of megakaryocytes in the pathogenesis of PMF.
Changes: Renewal Awarded

R01CA101774 (Crispino) 07/01/03 - 04/30/22 1.80 Calendar
NIH/NCI $213,750
Mechanisms of Leukemogenesis in Down Syndrome
The goals of this project are to 11) Determine the role of DYRK1A and its substrate STAT3 in leukemia in children with DS, and 2) Define the contributions of CHAF1B to normal and malignant hematopoiesis.
Changes: Renewal Awarded

R01DK101329 (Crispino) 09/18/13 – 07/31/18 1.80 Calendar
NIH/NIDDK $217,500
GATA1 Mutation in Defective Erythropoiesis
The aims of this project are to 1) Correlate chromatin occupancy of GATA1s with gene expression defects in primary GATA1s knock-in erythroid progenitor cells to identify key direct target genes that are dysregulated;
2) Investigate the consequences of the GATA1s mutation on erythroid specification and differentiation; and
3) Determine if loss of the N-terminus reduces the interaction with essential cofactors and in turn affects their chromatin occupancy
Changes since last reporting period: None

R6480-15 (Crispino) 10/01/14 - 08/31/18 0.60 Calendar
Leukemia and Lymphoma Society $270,027
MLN8237, an Aurora A kinase inhibitor, for the treatment of myeloid malignancies

This is a grant to sponsor a Phase I clinical study of MLN8237 in patients with Acute Megakaryoblastic Leukemia and Primary Myelofibrosis. The grant also supports correlative studies to identify biomarkers of response.
Changes since last reporting period: None

2013412 (Crispino/Israeli) 10/01/14 - 09/20/18 0.06 Calendar
United States-Israel Binational Science Foundation $6,660
Structure function analysis of ERG and GATA1 meg-erythroid development and leukemia
The goal of this study is to better understand the transcriptional regulation governed by GATA1 and ERG in hematopoiesis. This grant primarily supports travel for Dr. Crispino to visit Dr. Izraeli in Israel to enrich the collaboration.
Changes since last reporting period: Awarded

9001-16 (Crispino) 10/01/15 - 09/30/18 0.60 Calendar
Leukemia & Lymphoma Society $133,333
Identification of pathways that promote transformation of the MPNs to ML
The goal of this proposal is to identify tumor suppressor genes whose loss drives the transformation of primary myelofibrosis to acute myeloid leukemia.
Changes since last reporting period: None

Research Grant (Crispino) 07/01/16 - 06/30/18 0.12 Calendar
Rally Foundation $50,000
DYRK1A is a novel target in B cell leukemia
The aims of this grant are to perform pre-clinical studies of a novel DYRK1A kinase inhibitors in DS pre-B cell acute lymphoblastic leukemia.
Changes since last reporting period: None

Research Innovation Challenge Award (Crispino) 10/01/17-09/30/18
Robert H. Lurie Comprehensive Cancer Center $50,000
Novel insights into the biology and development of new therapeutics for MLL rearranged leukemia
This internal award supports research by Drs. Crispino, Shilatifard, Kelleher and Ntziachristos to develop new strategies to treat MLL-rearranged leukemia, including development of CHAF1B inhibitors.
Changes since last reporting period: Awarded

Research Grant (Crispino) 07/07/14-06/30/18 0.12 Calendar
Samuel Waxman Cancer Research Foundation $20,000
Novel Approaches to Targeting Megakaryocytic Malignancies
The aims of this grant are to study the relationship between GATA1 and ERG in acute leukemia in children with and without Down syndrome.
Changes since last reporting period: Awarded

P30CA060553 (Platanias) 08/01/13 - 07/31/18 1.80 Calendar
NIH/NCI $35,188
The Robert H. Lurie Comprehensive Cancer Center
The goals of this Cancer Center Support Grant are to conduct and support cancer research and to integrate cancer-related research throughout the university; to coordinate and integrate cancer-related activities of the University including community outreach initiatives; to develop and conduct cancer education programs; to promote and participate in state-of-the-are care of cancer patients at the affiliated hospitals of the McGaw Medical Center of Northwestern University and; to develop and implement the initiatives

in cancer prevention and control research. These goals are accomplished through the activities of the 9 establish programs and 15 shared resources.
Role: Associate Director for Education & Training
Changes since last reporting period: None

U54CA193419 (O'Halloran/Licht) 05/19/15 – 04/30/20 0.49 Calendar
NIH/NCI $9,000
Spatio-Temporal Organization of Chromatin and Information Transfer in Cancer
Project 3: Mechanisms of Nuclei Chromosomes and Chromatin in Cancer (Marko)
The goal of Project 3 will be a comprehensive study of how chromatin and chromosomes are remodeled in cancer cells relative to normal cells, and its research will be tightly linked to Project 1 and Project 2 via parallel studies of the same cell lines, use of the physical assays developed in Project 3, and through cooperation on development of siRNA and CRISPR methodologies.
Role: Co-Investigator
Changes since last reporting period: None

T32CA009560-26 (Green) 07/01/07-06/30/22 0.6 Calendar
NIH/NCI $294,746
Carcinogenesis Training Program
This award supports the pre-doctoral Carcinogenesis Training Program at Northwestern University, and currently supports 9 students. The goal of this program is to provide comprehensive and rigorous research training in cancer biology.
Role: Co-PI
Changes since last reporting period: Renewal Awarded

The following projects have ended:
Award Ltr 6/28/12 (Crispino) 07/01/08 - 06/30/17 0.12 Calendar
Waxman Foundation $20,000
The GATA-ERG axis in myeloid malignancies.
The goal of this project is to determine how GATA, GATA2, and ERG work together to drive the abnormal growth of a subset of blood cells named myeloid cells. Mouse models and patient data will be used to better understand the process of leukemia development and to design new strategies to treat this disease.

Research Grant (Crispino) 10/01/15 – 09/30/17 0.12 Calendar
Heartland Blood Centers $225,000
Role of cohesion mutations in Acute Myelogenous Leukemia
The goals of this project are to study the contribution of cohesin mutations to hematopoiesis and AML, examine the connection between loss cohesin or CTCF and trisomy 21, and to elucidate the molecular mechanism for cohesin and CTCF involvement in progression to DS-AMKL

What other organizations were involved as partners?
Nothing to Report

SPECIAL REPORTING REQUIREMENTS
Not Applicable

APPENDICES – please see following pages

1. Manuscript: **Age-related inflammatory bone marrow microenvironment induces ineffective erythropoiesis mimicking del(5q) MDS**. Yang Mei[1], Baobing Zhao[1], Ashley A Basiorka[2], Jing Yang[1], Lan Cao[1,3], Jingxin Zhang[1], Alan List[2,4], and Peng Ji[1]
2. Figures

Age-related inflammatory bone marrow microenvironment induces ineffective erythropoiesis mimicking del(5q) MDS

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Abstract

Anemia is characteristic of myelodysplastic syndromes (MDS). The mechanisms of anemia in MDS are unclear. Using a mouse genetic approach, here we show that dual deficiency of mDia1 and miR-146a, encoded on chromosome 5q and commonly deleted in MDS (del(5q) MDS), causes an aged-related anemia and ineffective erythropoiesis mimicking human MDS. We demonstrate that the ageing bone marrow microenvironment is important for the development of ineffective erythropoiesis in these mice. Damage-associated molecular pattern molecules (DAMPs), whose levels increase in ageing bone marrow, induced TNFα and IL-6 upregulation in myeloid-derived suppressor cells (MDSCs) in mDia1/miR-146a double knockout mice. Mechanistically, we reveal that pathologic levels of TNFα and IL-6 inhibit erythroid colony formation and differentially affect terminal erythropoiesis through reactive oxygen species-induced caspase-3 activation and apoptosis. Treatment of the mDia1/miR-146a double knockout mice with all-trans retinoic acid, which promoted the differentiation of MDSCs and ameliorated the inflammatory bone marrow microenvironment, significantly rescued anemia and ineffective erythropoiesis. Our study underscores the dual roles of the ageing microenvironment and genetic abnormalities in the pathogenesis of ineffective erythropoiesis in del(5q) MDS.

Introduction

Myelodysplastic syndromes (MDS) are age-related bone marrow malignancies characterized by dysplastic and ineffective production of myeloid cells and risk of developing acute myeloid leukemia (AML). Clinically, a great majority of patients with MDS have anemia caused by ineffective production of erythroid cells at different stages of erythropoiesis. The pathogenesis of ineffective erythropoiesis in MDS is unclear, which can be attributed to the heterogeneous nature of many genetic and molecular abnormalities involved in the development of MDS as well as micro-environmental factors. One of the most common cytogenetic defects in MDS is the heterozygous interstitial deletion of chromosome 5q (del(5q)). There are two common deleted regions (CDRs) identified on 5q: a distal locus that is often deleted in 5q- syndrome with good prognosis and a proximal locus deleted in patients with higher risk of MDS[1,2]. Refractory anemia is a characteristic feature of del(5q) MDS[3]. Recent studies have shown that haploinsufficiency of *Rps14* on the distal locus of the CDRs blocks erythroid differentiation through upregulation of p53 and its downstream genes *S100a8* and *S100a9*[4]. Whether deficiencies of other genes on chromosome 5q, and the ageing bone marrow microenvironment, are involved in ineffective erythropoiesis is unknown.

Growing evidence reveals that deregulation of innate immune responses is also involved in the pathogenesis of del(5q) MDS[5-11]. Concurrent loss of miR-145 and miR-146a, which are located at the distal region of 5q, leads to the dysplastic phenotype in megakaryocytes by the upregulation of their downstream target tumor necrosis factor receptor-associated factor-6 (TRAF6)[11]. Subsequent studies using miR-146a knockout mice show that miR-146a serves as a brake on inflammation and regulates

myeloproliferation and oncogenic transformation[12]. Our previous work shows that mDia1, whose encoding gene is flanked by the two CDRs on 5q, is significantly decreased in del(5q) MDS $CD34^+$ cells. mDia1 heterozygous and knockout mice developed age-related neutropenia and myeloid dysplasia mimicking human MDS[5]. Mechanistically, CD14 is aberrantly overexpressed on granulocytes, leading to a hypersensitive innate immune response to lipopolysaccharide (LPS) stimuli. A more recent study illustrates that loss of another 5q gene, *Tifab*, alters hematopoiesis through derepression of Toll-like receptor-TRAF6 pathway, leading to ineffective hematopoiesis and cytopenia[7]. In these studies, leukopenia, including neutropenia, is commonly observed, which demonstrates that deregulation of the innate immune signaling is involved in myeloid dysplasia. However, anemia is mild or not observed in these models, indicating that either loss of these genes are not essential for the development of ineffective erythropoiesis, or combined deficiencies, as those in del(5q) MDS, are required for the clinical manifestation of anemia.

In this study, we generate a mouse model with concurrent deletion of mDia1 and miR-146a, both are encoded by genes on chromosome 5q and involved in the repression of TLR-TRAF6 pathway. Mice with mDia1/miR-146a double knockout develop age-related anemia and ineffective erythropoiesis. Our study illustrates the importance of the age-related inflammatory bone marrow microenvironment in the pathogenesis of ineffective erythropoiesis and anemia in del(5q) MDS.

Methods

Experimental methods are in the supplemental materials.

Results

Dual deficiency of mDia1 and miR-146a causes age-related anemia in mice.

To characterize the extent of pathologically activated innate immune responses on the development of anemia in del(5q) MDS, we crossed the mDia1 knockout mice with miR-146a knockout mice and generated mDia1/miR-146a double knockout (hereafter DKO) mice. We monitored the complete blood counts in these mice over one year. mDia1 KO mice showed no anemia and miR-146a KO mice had mild anemia compared to the DWT control mice. In comparison, the DKO mice developed significant age-related anemia starting at 7 months, which became worse with age (**Figure 1a**). The DKO mice also displayed thrombocytopenia compared to single KO and DWT control mice. Both DKO and mDia1 single KO mice developed neutropenia (**Figure 1a**), which is consistent with previous findings[5]. Morphologic examination of the peripheral blood in the aged DKO mice showed severe anemia with anisopoikilocytosis including hypochromatic cells, Howell-Jolly bodies, and increased reticulocyte count (**Supplementary Figure 1a and b**).

We next examined the bone marrow of the one-year old mice to determine the cause of anemia. Gross observation showed pale bone and bone marrow cell aspirate from DKO mice and to a lesser extent from miR-146a knockout mice (**Supplementary Figure 1c**). We reasoned that the pale appearance of the DKO bone marrow was due to the

decreased erythroid population. Accordingly, total bone marrow cells and Ter119 (a maturing erythroid marker) positive erythroid cells were significantly decreased in DKO mice compared to wild type and single KO littermate controls, confirming our hypothesis (**Figure 1b and 1c**). The DKO mice also exhibited increased lethality around one year old, likely due to a life-threatening anemia (**Figure 1d**).

To further dissect the aberrancies in erythroid cell differentiation, we next performed a flow cytometric analysis using CD44 and forward scatter to divide the erythroid cells into different developmental stages[13]. The percentage of nucleated erythroblasts in the aged (>1 y old) DKO bone marrow was significantly decreased compared to littermate controls (**Supplementary Figure 1d and Figure 1e**). miR-146a KO mice also showed slightly decreased percentage of nucleated erythroblasts (**Supplementary Figure 1d and Figure 1e**). When the absolute number of erythroblasts was calculated from leg bones, aged miR-146a knockout mice showed a significant decrease, and erythroblasts from aged DKO mice further decreased compared to miR-146a knockout mice (**Figure 1f**).

Unlike patients with MDS, mice with anemia often show compensatory erythropoiesis evidenced by splenomegaly[14]. As expected, the old DKO mouse showed a dramatically increased spleen size compared to wild type or single knockout littermate controls (**Supplementary Figure 2a and b**). The percentage of splenic Ter119 positive erythroid cells increased by 20-30% in DKO mice due to stress erythropoiesis[15-17]. The percentages of lymphocytes were significantly reduced (**Supplementary Figure 2c**). We next examined different stages of terminal erythropoiesis in the spleen. Unlike the bone marrow, terminal erythropoiesis in the spleen did not show defects in various stages of

differentiation (**Supplementary Figure 2d**), These data suggest that ineffective erythropoiesis in the bone marrow is compensated by splenic stress erythropoiesis, likely protecting the DKO mice from early death induced by severe anemia.

Age-related bone marrow microenvironment contributes to the development of anemia.

Both mDia1 and miR-146a knockout mice show age-related increased secretion of pro-inflammatory cytokines, mostly TNF-α and IL-6[5,11,12]. To determine whether the defects of erythroid population are erythroid cell intrinsic or due to the inflammatory microenvironment, we performed two groups of transplantation experiments. In these experiments, bone marrow mononuclear cells from aged (> 1-year old) DKO mice or age matched DWT littermate controls were transplanted into either young (2-month old) or aged (> 1-year old) recipient mice (**Figure 2a**). Like the non-transplanted aged DKO mice, these transplanted mice exhibited neutropenia early post-transplantation due to the cell-intrinsic effect of mDia1 deficiency, as well as worsening thrombocytopenia (**Figure 2b**). The young recipient mice showed no significant differences in red cell indices between DKO and DWT transplanted mice until 6-month post-transplantation when RBC count, hemoglobin, and hematocrit began declining (**Figure 2c**). The age when anemia manifested in these transplanted mice was similar to that of the regular DKO mice (**Figure 1a**), implicating an age-related microenvironment in the development of anemia.

Old (> 1-year of age) recipient mice transplanted with old DWT or DKO bone marrow cells showed initial anemia due to their age. Those transplanted with DWT bone marrow

cells gradually recovered from transplantation stress. However, the old recipient mice transplanted with old DKO bone marrow cells exhibited persistent anemia starting from one month post-transplantation (**Figure 2d**). The old recipient mice transplanted with old DKO bone marrow cells also exhibited rapid lethality compared to the old DWT bone marrow transplanted controls (**Figure 2e**). When we analyzed the bone marrow of the old recipient mice from both groups of the transplantation experiments, we found ineffective erythropoiesis at different stages of red cell development similar to the non-transplanted old DKO mice (**Figure 2f**). Furthermore, when we transplanted young DKO bone marrow cells into old recipient mice (> 1-year of age), the recipient mice failed to recover from anemia compared to DWT transplanted ones (**Figure 2g**). Taken together, these results indicate that the age-related bone marrow microenvironment plays an important role in the development of ineffective erythropoiesis and anemia.

Since both mDia1 and miR-146a are involved in innate immune signaling, we asked whether chronic immune stimulation through LPS would accelerate the development of ineffective erythropoiesis. Unlike the untreated DKO mice that developed anemia and ineffective erythropoiesis at 7 months of age, the LPS treated DKO mice started to show anemia at 4 months together with thrombocytopenia and neutropenia (**Supplementary Figure 3a**). The absolute number of Ter119 positive erythroid cells in these LPS challenged DKO mice was also significantly decreased at 4 months of age (**Supplementary Figure 3b**). Like the old DKO mice, erythroblasts at various developmental stages were significantly decreased compared to the DWT and single knockout controls (**Supplementary Figure 3c**). These mice also showed prominent splenomegaly, compensating for ineffective erythropoiesis within the bone marrow (**Supplementary Figure 3d**). These results support the role of an inflammatory bone

marrow microenvironment in the development of ineffective erythropoiesis and anemia in the context of concurrent loss of mDia1 and miR-146a in the hematopoietic populations.

Proinflammatory cytokines are over-produced in the bone marrow myeloid-derived suppressor cells in aged mDia1/miR-146a double knockout mice.

With the dramatic decrease of the erythroid population in the bone marrow of the aged DKO mice, the relative percentage of Gr1 and Mac1 double positive myeloid cells was significantly increased (**Figure 3a and b**). The absolute number of myeloid cells was decreased in the bone marrow of DKO mice, albeit to a lesser extent than the decrease observed in the erythroid population (**Supplementary Figure 4a**). Consistent with the flow cytometric results, morphologic examination of the bone marrow from the old DKO mice showed marked erythroid hypoplasia with most of the cells in the myeloid lineage (**Figure 3c**). Many of these myeloid cells were immature appearing with large cell size measured by forward scatter by flow cytometry (**Figure 3d and e**). However, the DKO mice did not develop acute leukemia demonstrated by their decreased numbers of lineage negative cells as well as stem and progenitor cells (**Supplementary Figure 4b-e**).

A previous report demonstrated that myeloid derived suppressor cells (MDSCs), which are characteristically Gr1 and Mac1 double positive, immature appearing, and immunosuppressive, are enriched in the bone marrow in MDS patients[18]. Indeed, the immature appearing myeloid cells in the old DKO mice fulfill these criteria and blocked T cell activation when co-cultured with CD4 positive T cells *in vitro* (data not shown). Consistently, MDSCs, including the monocytic-MDSCs that were reported to have

stronger suppressive functions[19], were significantly increased in the peripheral blood of DKO mice. Consequently, the lymphoid population was significantly decreased (**Supplementary Figure 4f**).

The ageing bone marrow microenvironment is composed of increasing amounts of damage associated molecular patterns (DAMPs), which are potent inducers of pro-inflammatory cytokines production, especially in cells with sensitized innate immune signaling[8,9,20]. To analyze whether treatment of DAMPs could induce the over-production of pro-inflammatory cytokines in the MDSCs in the DKO mice *in vitro*, we purified Gr1 and Mac1 double positive cells from the bone marrow of young DKO mice and their littermate controls. These cells were treated with DAMPs prepared through repetitive freeze and thaw cycles of wild-type bone marrow cells. Indeed, treatment with DAMPs induced over-production of TNF-α and IL-6 in the bone marrow-derived myeloid cells from each group of mice, with levels much higher in the MDSCs from DKO cells (**Figure 3f**). Particularly, IL-6 presented the most profound (up to 200-fold change) up-regulations, suggesting a more crucial role of IL-6 in the pathogenesis in DKO mice.

We next analyzed the serum levels of inflammatory cytokines using a mouse cytokine/chemokine panel. As expected, the serum levels of IL-6 and TNFα were significantly increased in old DKO mice compared to age-matched DWT and single knockout controls (**Supplementary Figure 5**), which was further confirmed by conventional ELISA assay (**Figure 3g**). The level of IL-10, which is highly expressed by MDSCs[21], was also significantly upregulated in DKO mice (**Supplementary Figure 5**), consisting with the increased MDSCs in DKO mice. These results collectively indicate

that bone marrow microenvironment-mediated ineffective erythropoiesis in DKO mice could be related to the over-secretion of pro-inflammatory cytokines.

TNFα and IL-6 negatively affect erythropoiesis through different mechanisms.

Pro-inflammatory cytokines are known to be involved in anemia of chronic diseases (ACD) through upregulation of liver hepcidin, which limits the availability of iron to the developing erythroblasts[22-24]. However, the increased cytokine levels did not induce hepcidin upregulation in hepatocytes from aged DKO mice compared to the control mice (**Figure 4a**), indicating that ineffective erythropoiesis in aged DKO mice was likely hepcidin-independent. Next, we investigated whether the pro-inflammatory cytokines TNFα and IL-6 directly affect erythropoiesis *in vitro*. We first purified the lineage negative bone marrow cells from old (> 1 year of age) DWT and DKO mice and cultured them in erythropoietin-containing medium for three days. No statistically significant differences were observed in their differentiation, as quantified by CD71 and Ter119 double positive cells (**Figure 4b**). Similar findings were obtained from young DKO and DWT bone marrow lineage negative cells (data not shown), which further supports that ineffective erythropoiesis is erythroid cell non-autonomous. Therefore, we used DWT bone marrow cells to determine how pro-inflammatory cytokines affect various stages of erythropoiesis. Previous studies have shown inhibitory effects of pro-inflammatory cytokines in erythropoiesis[20]. However, the mechanisms of cytokine-mediated inhibition of erythropoiesis, the stages at which the inhibitory effects are conferred, and whether there are any differences between TNFα and IL-6 in inducing ineffective erythropoiesis, are unknown.

To answer these questions, we purified bone marrow cells from young DWT bone marrow and cultured the cells in methocellulose with erythropoietin and increasing amounts of TNFα and IL-6. Both TNFα and IL-6 induced a dose-dependent decrease in Colony-Forming Unit- Erythroid (CFU-E) colony formation (**Figure 4c**), demonstrating that increasing levels of both cytokines affect the early stage of erythropoiesis. After the CFU-E stage, the erythroid cells undergo terminal erythropoiesis that can be monitored by flow cytometry. We next determined the differentiation of DWT bone marrow lineage negative cells cultured with erythropoietin and increasing amount of TNFα and IL-6. These cytokines did not significantly affect cell differentiation, as measured by Ter119 positive cells. Instead, high levels of both TNFα and IL-6 induced increased Ter119 positive cells on day 1 (**Supplementary Figure 6a**), possibly contributing to the depletion of erythroid progenitor cells.

We next found that IL-6 induced a dose-dependent increase in annexin V positive apoptotic cells at different stages of terminal erythropoiesis. TNFα-mediated apoptosis (annexin V positive) was only observed on day 3 (**Figure 4d**). TNFα also induced mild necrosis (annexin V negative and PI positive) on day 2 and 3 whereas IL-6 treated cells showed no increased necrosis compared to the vehicle controls (**Supplementary Figure 6b**). Pathologic levels of both IL-6 and TNFα induced downregulation of GATA1 (**Supplementary Figure 6c and d**), a critical transcription factor in erythropoiesis[25]. TNFα caused the most significant downregulation of GATA1 from the mRNA level (**Supplementary Figure 6e**), which further reduced the expression of the downstream targets of GATA1, including hemoglobin and Fog-1 (data not shown).

Reactive oxygen species (ROS) induced by pro-inflammatory cytokines are known to promote apoptosis[26]. Our previous report demonstrated that ROS presents at the highest level in the early stages of terminal erythropoiesis, which corresponds to day 1 of the erythroid differentiation culture system[27]. ROS levels gradually decreased on day 2 and 3 in untreated cells (**Figure 4e**). Administration of IL-6 led to a dose-dependent increase of ROS beyond the physiologic level, which was most prominent on day 1. In contrast, TNFα treated cells did not show increased ROS levels (**Figure 4e**). Consistent with the role of ROS in the activation of caspase-3, IL-6 treated cells exhibited a dose-dependent increase of active caspase-3, correlating with their apoptotic cell death. No increased activation of caspase-3 was observed in TNFα-treated cells (**Figure 4f**). Cell cycle analysis showed that the percentage of S and G2/M phases were slightly increased with the treatment of IL-6 and TNFα on day 1 (**Supplemental Figure 6f**), which could contribute to the increased percentage of Ter119+ cells (**Supplemental Figure 6a**).

To further confirm the role of IL-6-induced ROS in the activation of caspase-3, we treated the IL-6 (100 ng/ml) challenged bone marrow lineage negative progenitor cells with increasing amount of ROS scavenger, N-acetyl-cysteine (NAC). Indeed, NAC dose-dependently rescued the increased ROS formation and activation of caspase-3 when the lineage negative cells underwent differentiation to erythroid cells (**Figure 4g**). Taken together, these results demonstrate that TNFα and IL-6 negatively affect erythropoiesis through different mechanisms. Both cytokines compromise CFU-E colony formation at higher levels. In addition, IL-6 also induces ROS-mediated activation of caspase-3 and apoptosis, which is not observed with TNFα.

We next determine whether ROS and apoptosis were increased *in vivo* in erythroid cells from DKO mice. Like the *in vitro* cytokine treated erythroid cells, ROS levels were markedly elevated beyond the physiologic level at different stages of terminal erythropoiesis in DKO mice compared to DWT controls (**Figure 4h**). Annexin V positive apoptotic cells were also significantly increased in the Ter119 positive erythroid cells from DKO mice compared to DWT or single knockouts (**Supplemental Figure 6g and h**). As expected, different stages of terminal erythroblasts showed increased apoptotic cells, as measured by activation of caspase-3 (**Figure 4i**).

Treatment of all-trans retinoic acid rescues anemia and ineffective erythropoiesis through amelioration of MDSC-induced bone marrow inflammatory microenvironment in DKO mice.

Having demonstrated that the Gr1/Mac1 double positive MDSCs secreted a large amount of TNFα and IL-6, we ask whether reducing the amount of immature MDSCs through the promotion of their differentiation would rescue anemia and ineffective erythropoiesis. To this end, we treated the old (>1 year of age) DKO mice with all-trans retinoic acid (ATRA), which induces the differentiation of immature myeloid cells, for 3 consecutive doses. Complete blood count was performed after the treatment, which showed significant rescue of anemia (**Figure 5a**). ATRA also significantly reverted the ineffective erythropoiesis in the bone marrow demonstrated by the recovered Ter119 positive cells in percentage and total number (**Figure 5b and c**).

We further analyzed the ATRA treated DKO mice and found that serum TNFα and IL-6 was significantly decreased (**Figure 5d**). The mRNA levels of TNFα and IL-6 were also markedly decreased almost to the control level in the DKO Gr1+/Mac1+ MDSCs treated with ATRA after DAMP stimulation *in vitro* (**Figure 5e**). Consistent with the decreased pro-inflammatory cytokines, the apoptotic erythroid cells were also dramatically reduced in ATRA treated DKO mice (**Figure 5f**). Indeed, ATRA not only rescued ineffective erythropoiesis in DKO mice, it also reverted the T cell inhibitory effects by MDSCs (**Figure 5g**). Bone marrow analysis showed that the mean size of MDSCs, including both granulocytic and monocytic-MDSCs, were reduced. The percentage of more suppressive monocytic-MDSCs was also reduced, which is accompanied by the increased percentage of granulocytic MDSCs (**Figure 5h**).

***In vitro* IL-6 treated bone marrow erythroblasts share common gene expression profiles as the erythroblasts from DKO mice.**

To further elucidate the inhibitory effects of pro-inflammatory cytokine in the ineffective erythropoiesis in DKO mice, we performed microarray analyses of IL-6-challenged erythroblasts cultured *in vitro* as well as primary bone marrow erythroblasts at different developmental stages sorted from DWT and DKO mice. Compared to the controls, IL-6 treated erythroblasts on day 1 revealed fewer up- or down-regulated genes. However, IL-6 treated erythroblasts on day 2 showed more robust changes in gene expression up to 577 significant transcripts (**Figure 6a**). In the erythroblasts from DKO bone marrow, 409, 518, and 474 genes were found to be changed significantly in stages I, II+III, and IV, respectively (I=pro-erythroblasts, II+III=Basophilic and polychromatic erythroblasts, IV=Orthochromatic erythroblasts). *DIAPH1* (encoding mDia1) and *CD14* were down and

up-regulated respectively at all stages, which confirms the reliability of our microarray platforms (**Figure 6b**).

The commonly up-regulated genes shared among IL-6 treated and DKO erythroblast groups were identified and illustrated with a Venn diagram (**Figure 6c**). These genes were applied to a clustering analysis (**Supplementary Figure 7**). Consistent with the induction of apoptosis in IL-6 treated and DKO erythroblasts, genes involved in the apoptotic pathway were commonly up-regulated. We also found that the S100 proteins, including S100a4, S100a6 and S100a8, were also significantly increased (pointed out in Figure. 6a and b).

To comprehensively analyze the signaling pathways involved in the up-regulated genes, we performed a functional PANTHER Pathway analysis[28]. All five groups shared the same inflammation pathway mediated by chemokine and cytokine as the top-signaling pathway. Genes involved in oxidative stress response and apoptosis signaling pathway were also commonly upregulated (**Supplementary Figure 8**), which is consistent with the apoptotic cell death in these cells. We next perform a common pathway analysis using a Venn diagram between Day2 and other three DKO groups. Thirty pathways, including the apoptosis signaling pathway, were found to be commonly upregulated among IL-6 treated erythroblasts *in vitro* and DKO erythroblasts *in vivo* (**Figure 6d**). When these 30 pathways were further aligned by percentage of gene hits and number of hit genes, the inflammation pathway mediated by chemokine and cytokine was the most common pathway (**Figure 6e**), which further confirms that the ineffective erythropoiesis

in DKO mice is directly related to the IL-6-enriched inflammatory bone marrow environment.

Discussion

In this study, we reveal the significant roles of age-related inflammation in bone marrow microenvironment and its cooperative interaction with chromosome 5q deleted genes in the pathogenesis of ineffective erythropoiesis in MDS. Aged bone marrow accumulates DAMPs and PAMPs, which direct activation and expansion of MDSCs, especially those primed by deletion of mDia1 and miR-146a, for excess IL-6 and TNFa elaboration. These proinflammatory cytokines accumulate to a pathologic level and trigger the over-production of reactive oxygen species that initiate caspase-mediated erythroid cell death, or GATA1 degradation leading to the inhibition of terminal erythropoiesis. The apoptotic erythroid cells further produce DAMPs, including S100 proteins, which creates a positive feedback loop to enhance the inflammatory bone marrow microenvironment-mediated ineffective erythropoiesis (**Figure 6f**).

Deletions of 5q genes in del(5q) MDS are almost uniformly hemizygous. Although other genetic abnormalities in the retained 5q alleles have not been detected so far, recent studies indicate that many genes on the retained 5q alleles are epigenetically silenced[29,30]. In fact, epigenetic dysregulation is commonly seen in MDS leading to the loss of function of many tumor suppressor proteins[31]. Therefore, we employed the

mDia1 and miR-146a double knockout mouse model instead of the double heterozygous mice in this study. This approach also allows us to compare the current data with previous reports using miR-146 knockout mice to study myeloid neoplasms[32-34].

The Gr1 and Mac1 double positive myeloid cells in old DKO mice fulfill the criteria of MDSCs with their immature morphology and T cell suppressive activity. In our model, these MDSCs also potently suppress erythropoiesis. Therefore, the erythroid suppressive function represents a key pathologic feature of MDSCs as previously reported[10,18,21]. By targeting MDSCs through all-trans retinoic acid (ATRA), we found improved erythropoiesis in DKO mice. Mechanically, ATRA attenuated the cytokine up-regulation both *in vivo* and *in vitro*, through the promotion of cell differentiation evidenced by the reduced cell size of MDSCs post-ATRA treatment and the percentage of monocytic-MDSCs. In fact, several clinical trials using ATRA to treat MDS have been reported[35-37]. One report showed ATRA combined with erythropoietin was effective for the treatment of anemia in low risk MDS patients having failed erythropoietin alone[35]. Our study provides mechanistic insights showing that ATRA ameliorates anemia in MDS through reduction of MDSC-induced inflammatory bone marrow microenvironment.

TNFα and IL-6 are known to inhibit erythropoiesis[38]. However, the detailed mechanisms of how different cytokines are involved in ineffective erythropoiesis are unclear. One of the most commonly involved pathways in inflammation-related anemia is cytokine-mediated hepcidin upregulation in the liver, which negatively affects the availability of iron to the developing erythroblasts[24,39]. However, hepcidin is not increased in our DKO mouse model. Instead, our study revealed direct but distinct roles of TNFα and IL-6 in

the pathogenesis of ineffective erythropoiesis. Both cytokines blocked erythroid colony formation. After the CFU-E stage, IL-6 induced potent upregulation of reactive oxygen species and caspase-mediated cell death whereas TNFα mainly affects GATA1 protein levels. These results are also consistent with reported clinical observations that IL-6 and activated caspase-1 and caspase-3 are upregulated in MDS patients with refractory anemia[9,10].

IL-6 is upregulated in miR-146a knockout mice through increased TRAF6, a well-known target of miR-146a[32]. Our previous study shows that IL-6 and TNFα are also prone to be upregulated in mDia1 deficient mice when the mice are subject to innate immune stimulation, related to increased expression of CD14 on granulocytes[5]. Here we show that the combined deficiency of miR-146a and mDia1 leads to a profoundly increased elaboration of these cytokines, especially IL-6. This increase is most prominent in aged mice, which is consistent with the age-related changes in the bone marrow microenvironment. The level of IL-6 correlates with the level of ineffective erythropoiesis. In miR-146a/mDia1 double knockout mice, IL-6 is nearly four-fold higher than miR-146a knockout mice and 20-30-fold higher than the wild type or mDia1 knockout mice. Correspondingly, bone marrow erythroid cells are significantly reduced in miR-146a knockout mice and almost completely absent in miR-146a/mDia1 double knockout mice. These results are consistent with a more severe effect of IL-6 in erythropoiesis. Overall, our study highlights the significance of the bone marrow inflammatory microenvironment in the pathogenesis of ineffective erythropoiesis and anemia in del(5q) MDS.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Figure Legends

Figure 1. mDia1/miR-146a double knockout mice develop age-related anemia and ineffective erythropoiesis.

(**a**) Complete blood count of different time points of mDia1/miR-146a double wild type (DWT), mDia1 or miR-146a single knockout (KO), and double knockout (DKO) mice. DWT, n=6; miR-146a KO, n=6; mDia1 KO, n=5; DKO, n=7. (**b-c**) Flow cytometric analysis of bone marrow cells from indicated mice at one year old. Total bone marrow cells (b) and absolute $Ter119^+$ erythroid cells (c) from femur and tibia were presented. DWT, n=6; miR-146a KO, n=4; mDia1 KO, n=5; DKO, n=5. (**d**) Kaplan-Meier survival analysis of the indicated mice. DWT, n=14; miR-146a KO, n=8; mDia1 KO, n=6; DKO, n=11. Both males and females were included. (**e-f**) The $Ter119^+$ erythroid cells in c were analyzed by CD44 levels and forward scatter to define various developmental stages of erythroblasts (Pro-: proerythroblasts, Baso-: basophilic erythroblasts, Poly-: polychromatic erythroblasts, Ortho-: orthochromatic erythroblasts, reticulocytes (Retic-) and mature red blood cells (RBC)). Quantification of the percentages and absolute cell numbers of erythroblasts at each stage of differentiation were presented in e and f, respectively. DWT, n=6; miR-146a KO, n=4; mDia1 KO, n=5; DKO, n=5.

Figure 2. The aging bone marrow microenvironment plays an important role in the development of anemia and ineffective erythropoiesis.

(**a**) Schematic overview of the bone marrow transplantation experiments. (**b-c**) Post-transplant complete blood counts at the indicated time of recipient mice (2-month old when transplanted) transplanted with bone marrow cells from old DWT or DKO mice (> 1-year-old). N=5 in each group. (**d**) Post-transplant red cell indices at the indicated time of recipient mice (> 1-year-old when transplanted) transplanted with bone marrow cells

from old DWT or DKO mice (>1-year-old). N=10 in each group. (**e**) Kaplan-Meier survival analysis of the recipient mice in (d). N=18 in DWT; N=16 in DKO. (**f**) The bone marrow $Ter119^+$ erythroid cells from both sets of transplantation experiments were analyzed by CD44 levels and forward scatter to define various developmental stages of erythroblasts as in Figure 1f. N=3 in each group. (**g**) Post-transplant red cell indices at the indicated time of the recipient mice (> 1-year-old when transplanted) transplanted with bone marrow cells from young DWT or DKO mice (2-month-old). N=9 in DWT; N=7 in DKO.

Figure 3. Pro-inflammatory cytokines are over-produced by myeloid-derived suppressive cells in aged mDia1/miR-146a double knockout mice.

(**a-b**) Flow cytometric analysis of Mac1 and Gr1 positive cells in the bone marrow from indicated mice at one year old. The percentages of Gr1/Mac1 double positive cells from femur and tibia were presented in (b). DWT, n=6; miR-146a KO, n=4; mDia1 KO, n=5; DKO, n=5. (**c**) H&E stains of bone marrow sections of indicated mice. Arrows indicate erythroblasts. Scale bar: 50 μm. (**d**) Bone marrow smear prepared from the indicated mice and stained with Wright-Giemsa. Black arrows indicate immature-appearing myelocytes. Scale bar: 30 μm. (**e**) Statistical analysis of the size of immature-appearing MDSCs measured by flow cytometry forward scatter of Gr1/Mac1 double positive cells from indicated mice. Data were presented as min- to max-box and whisker plots. (**f**) Gr1/Mac1 double positive granulocytes from the indicated mice were purified and challenged with damage-associated molecular pattern proteins (DAMPs) (1:20 for 2 hour). The relative mRNA levels of TNFα and IL-6 were determinate by real-time PCR analysis. (**g**) Serum IL-6 and TNFα from indicated mice (> 1-year-old) were assayed by ELISA. DWT, n=6; miR-146a KO, n=4; mDia1 KO, n=5; DKO, n=6.

Figure 4. IL-6 and TNFα induce ineffective erythropoiesis *in vitro* through distinct mechanisms.

(**a**) Quantification of hepcidin mRNA levels in hepatocytes by real-time PCR. N=5 in each group. (**b**) Bone marrow lineage negative cells (Lin^-) from indicated mice were cultured in erythroid differentiation medium containing 2 U/ml erythropoietin. Cell differentiation was analyzed by flow cytometric analysis comparing the expression of transferrin receptor CD71 and Ter119. (**c**) CFU-E colony formation assay of bone marrow cells cultured in methylcellulose medium with 3 U/ml erythropoietin and increasing amounts of TNFα and IL-6. (**d**) Lin^- cells from C57BL/6 wild-type mice were purified and cultured in erythroid differentiation medium in the presence of increasing amounts of IL-6 and TNFα (50,100, and 200 ng/mL) for 3 days. The annexin V positive cells in the Ter119+ erythroid cells were quantified. (**e-f**) The ROS and active caspase-3 levels were assayed by flow cytometry in Ter119 positive cells from (d), and were shown in (e) and (f) respectively. (**g**) Lin^- cells were pre-treated with 100 ng/mL IL-6 in EPO medium for 24 hours. 1-20 μM NAC were then added directly into the medium and the cells were cultured for another 24 hours. The ROS levels and active caspase-3 in the cells were assayed by flow cytometric analysis. Relative levels of ROS and active caspase-3 levels were presented. All the data are shown as mean ± SEM and representative data from 3 independent experiments. NS: not significant. *P<0.05; **P<0.01; ***P<0.001. (**h**) Mean fluorescence intensity (MFI) of ROS positive cells were analyzed at different populations of the erythroblasts and ungated bone marrow erythroid populations in old mDia1/miR-146a DWT or DKO mice (> 1-year-old). DWT, n=8; DKO, n=5. (**i**) Mean percentages of apoptotic cells in different populations and ungated bone marrow erythroblasts from indicated mice (> 1-year-old). DWT, n=7; DKO, n=4.

Figure 5. Amelioration of anemia in DKO mice by targeting MDSCs with AT-RA treatment.

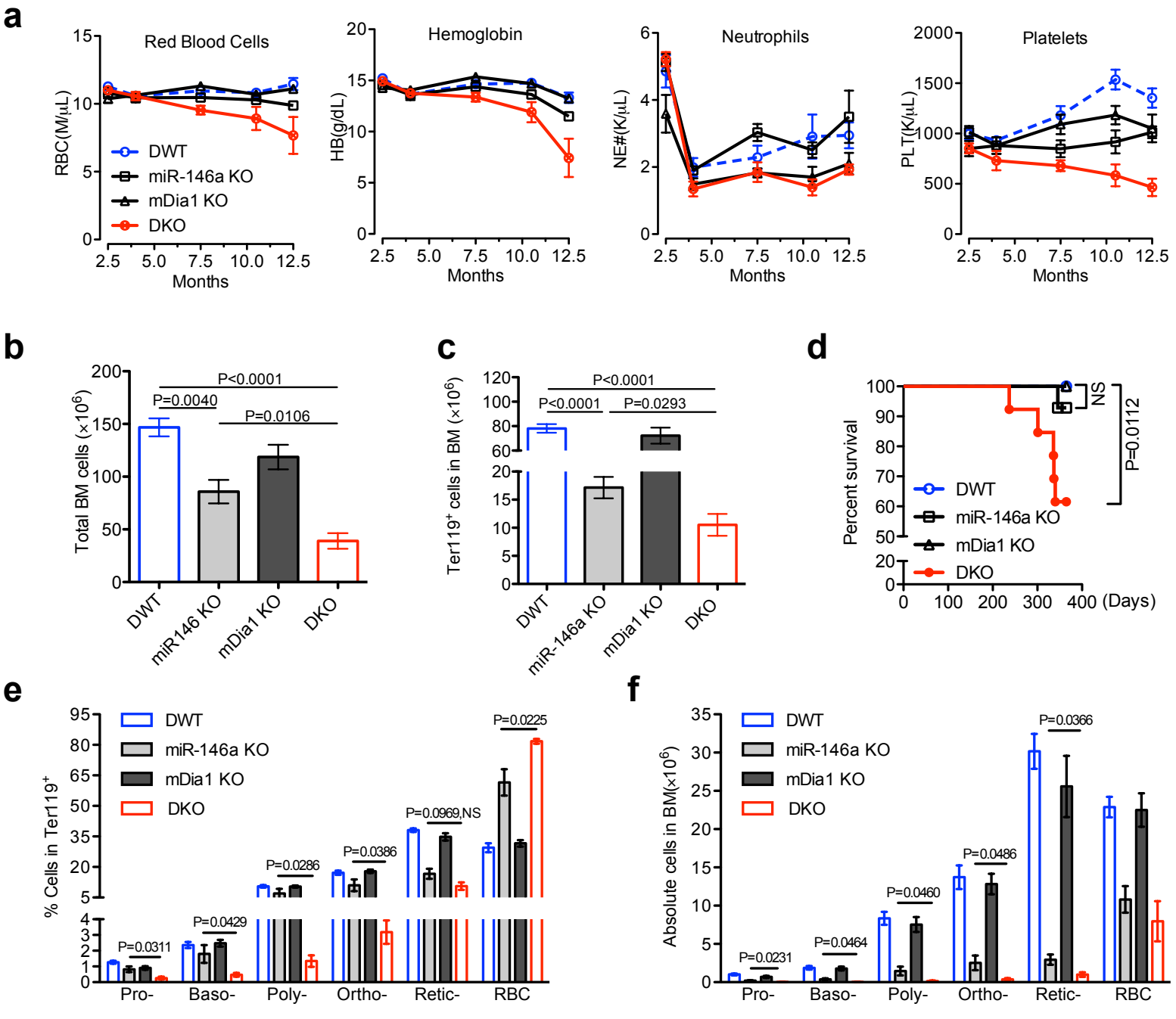
(**a**) DWT or DKO mice were intraperitoneally injected with 400 μg ATRA dissolved in 100 μl DMSO, or equal volume of vehicle control every two days for three doses. 48 hours after the last injection, red blood cell counts and hemoglobin levels were assayed. (**b-c**). The percentages of erythroid cells at different stages were examined by flow cytometry in Ter119 positive cells from the bone marrow and spleen from mice in (a). The absolute erythroblasts cell number was calculated in (c). (**d**) Serum TNFa and IL-6 cytokine levels were determined in mice with or without ATRA treatment. (**e**) Gr1 and Mac1 double positive MDSCs were purified from the indicated mice and challenged with 1:20 DAMPs for 2 hours *in vitro*. The mRNA levels of TNFa and IL-6 were assayed by real-time PCR using 18s rRNA as an internal control. (**f**) The apoptotic erythroblasts in the bone marrow and spleen from the indicated mice were analyzed by Annexin V and PI staining followed by flow cytometric analysis. (**g**) Gr1 and Mac1 double positive MDSCs were purified and co-cultured with cell proliferation dye labeled-splenic T cells at 1:1 ratio, the mean fluorescence intensity (MFI) were determined by flow cytometric assay after 48-hour stimulation by anti-CD3/CD28 coated Dynabeads. The proliferation rate was calculated as percentage decrease of MFI relative to cells without stimulation. (**h**) Granulocytic ($CD11b^{+}Ly6G^{+}Ly6C^{low}$) and monocytic ($CD11b^{+}Ly6G^{-}Ly6^{high}$) MDSCs from the bone marrow and peripheral blood of mice in (a) were assayed by flow cytometry. N=3-5 mice in each group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

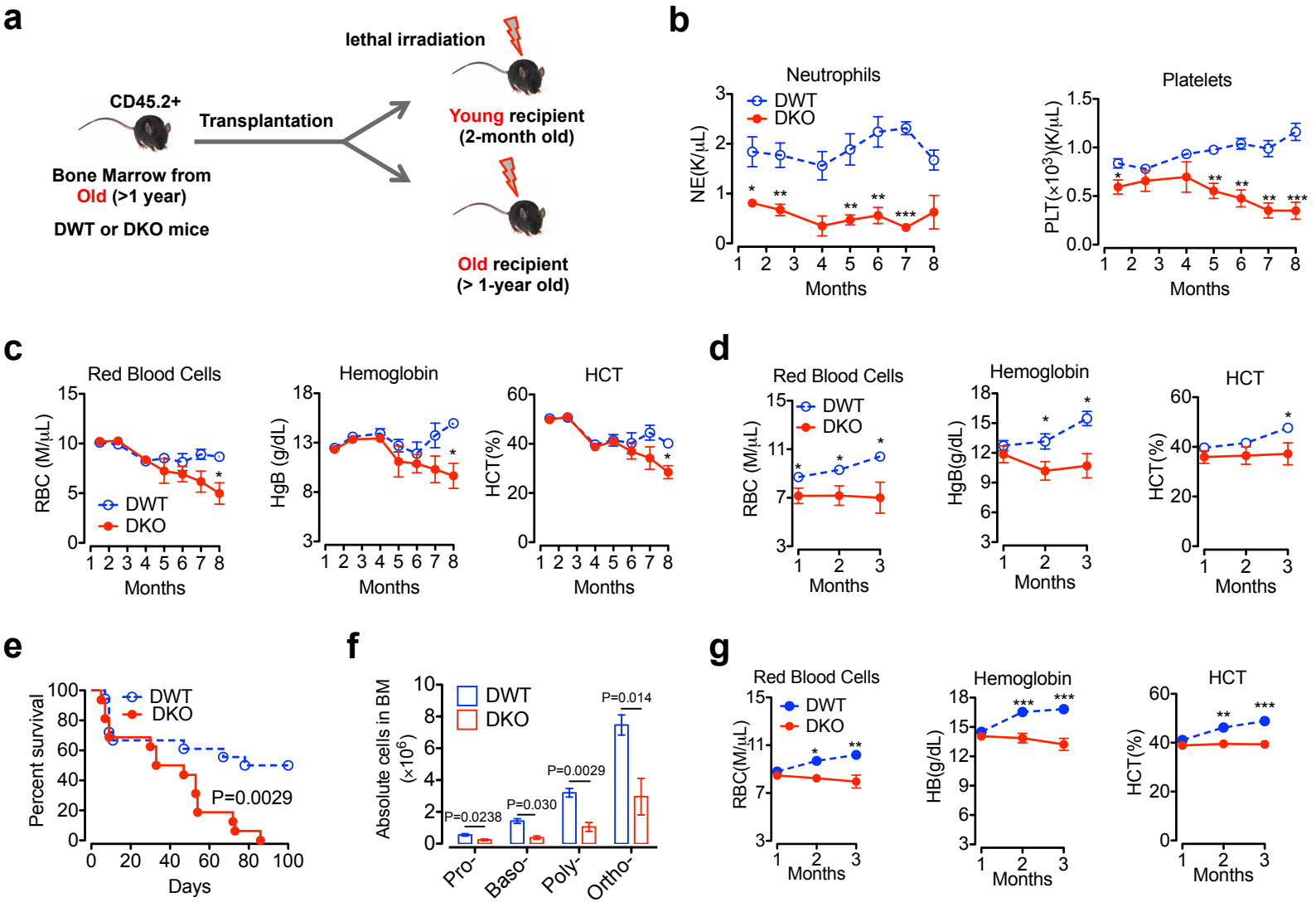
Figure 6. *In vitro* IL-6 treated bone marrow erythroblasts share the same gene expression profiles as the erythroblasts from DKO mice.

(**a-b**) Volcano plot of microarray data, as generated by clustering based on the genes

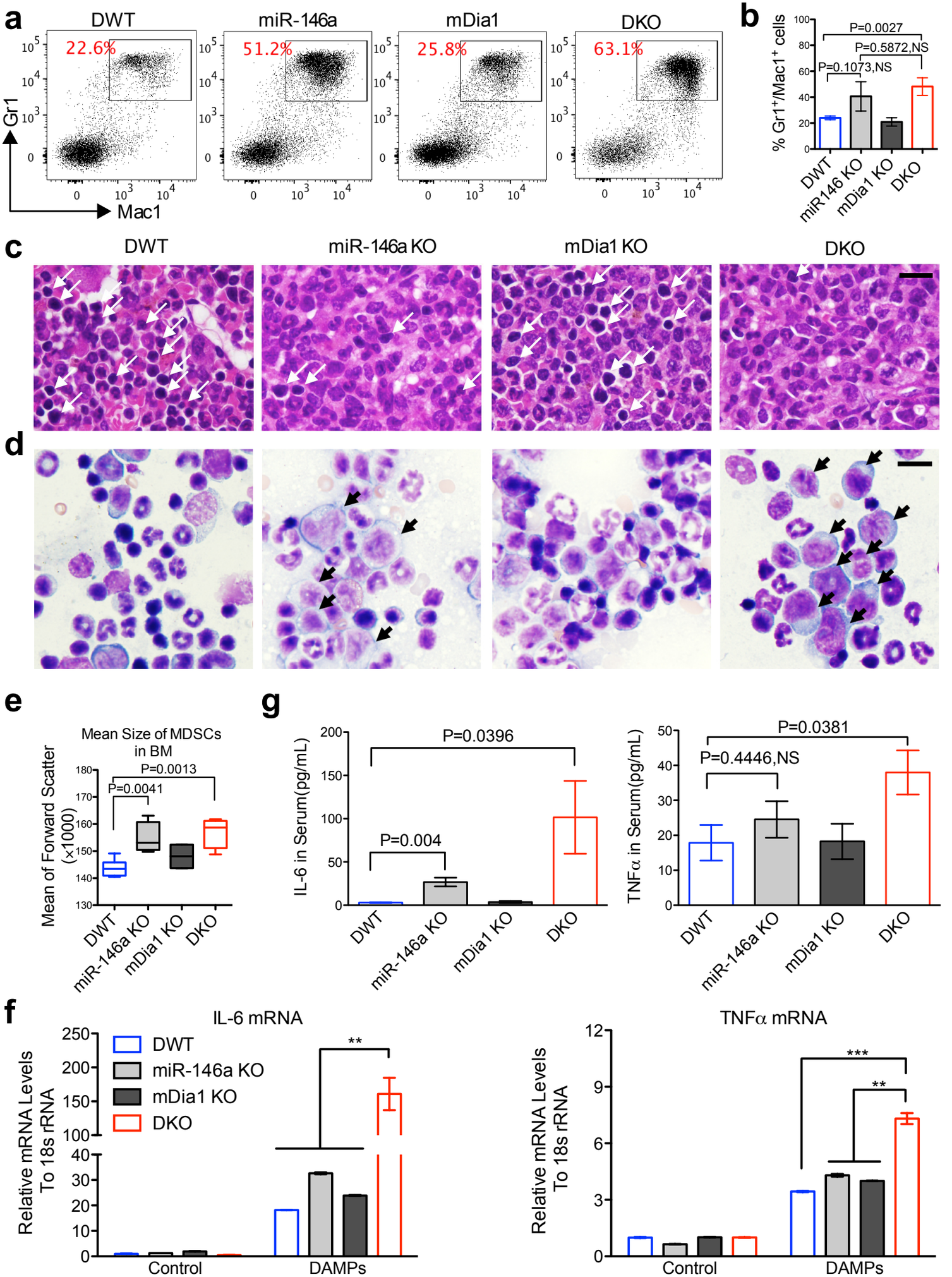
that were significantly enriched (red dots) or depleted (green dots) (fold change >2; ANOVA P<0.05) in IL-6-treated (a) or DKO erythroblasts (b) over their controls. Representative up or down-regulated genes are pointed out. I represents proerythroblasts, II+III represent basophilic and polychromatic erythroblasts, IV represents orthochromatic erythroblasts. (**c**) Venn diagram defining significantly up-regulated genes in in vitro IL-6 treated erythroblasts and erythroblasts from different developmental stages in DKO mice and compared with their corresponding controls. The numbers of overlapped genes are also shown. (**d**) Venn diagram showing the overlapping signaling pathways generated by PANTHER pathway analysis among the indicated groups (d). 30 signaling pathway were enriched both in Day 2 IL-6 treated erythroblasts and DKO erythroblasts. (**e**) The percentage of gene hits against total numbers of Pathway hits and gene numbers in specific pathway are illustrated by color scales. (**f**) Model of the age-related bone marrow microenvironment in the pathogenesis of ineffective erythropoiesis in del(5q) MDS.

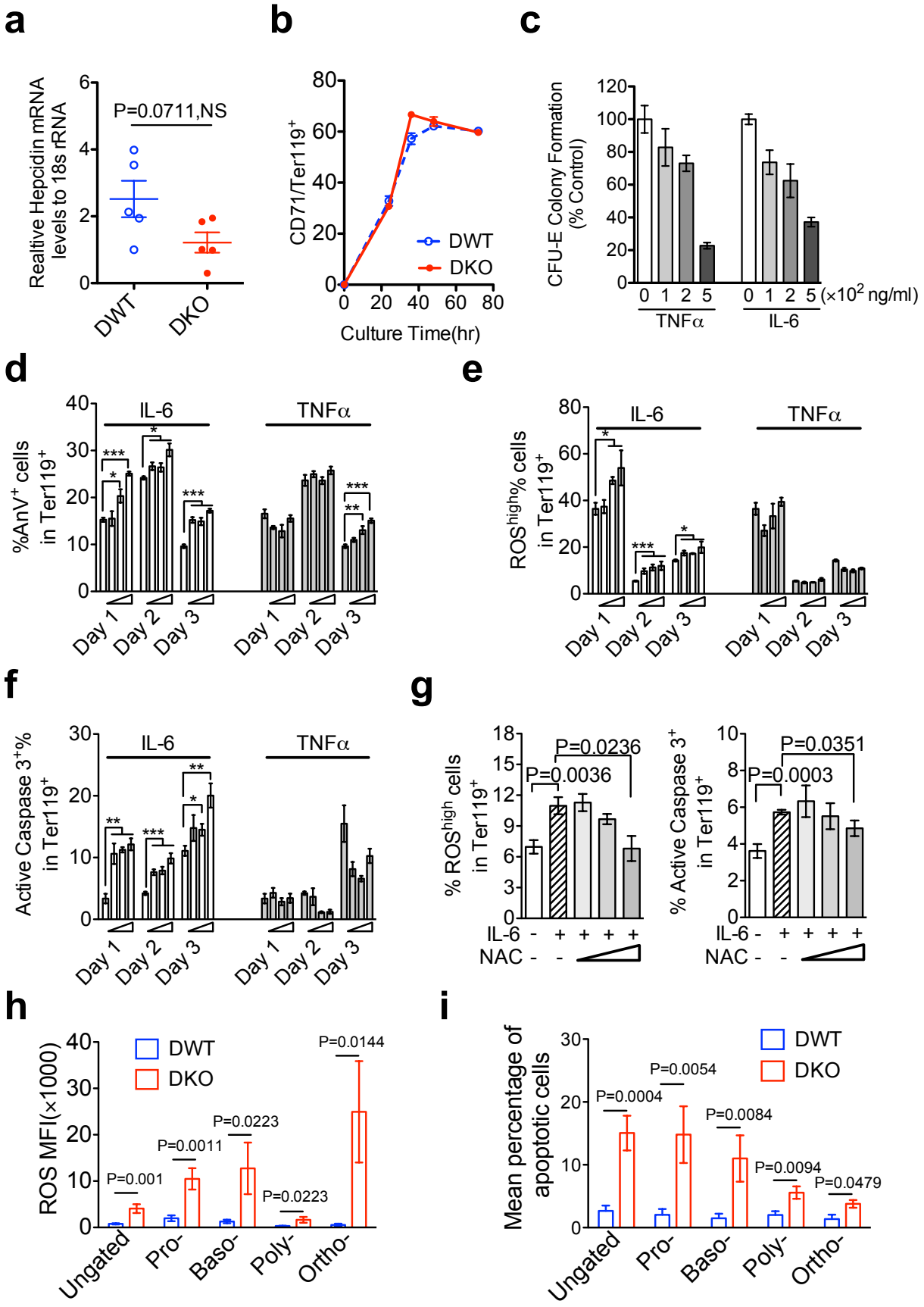
Mei et al. Figure 1

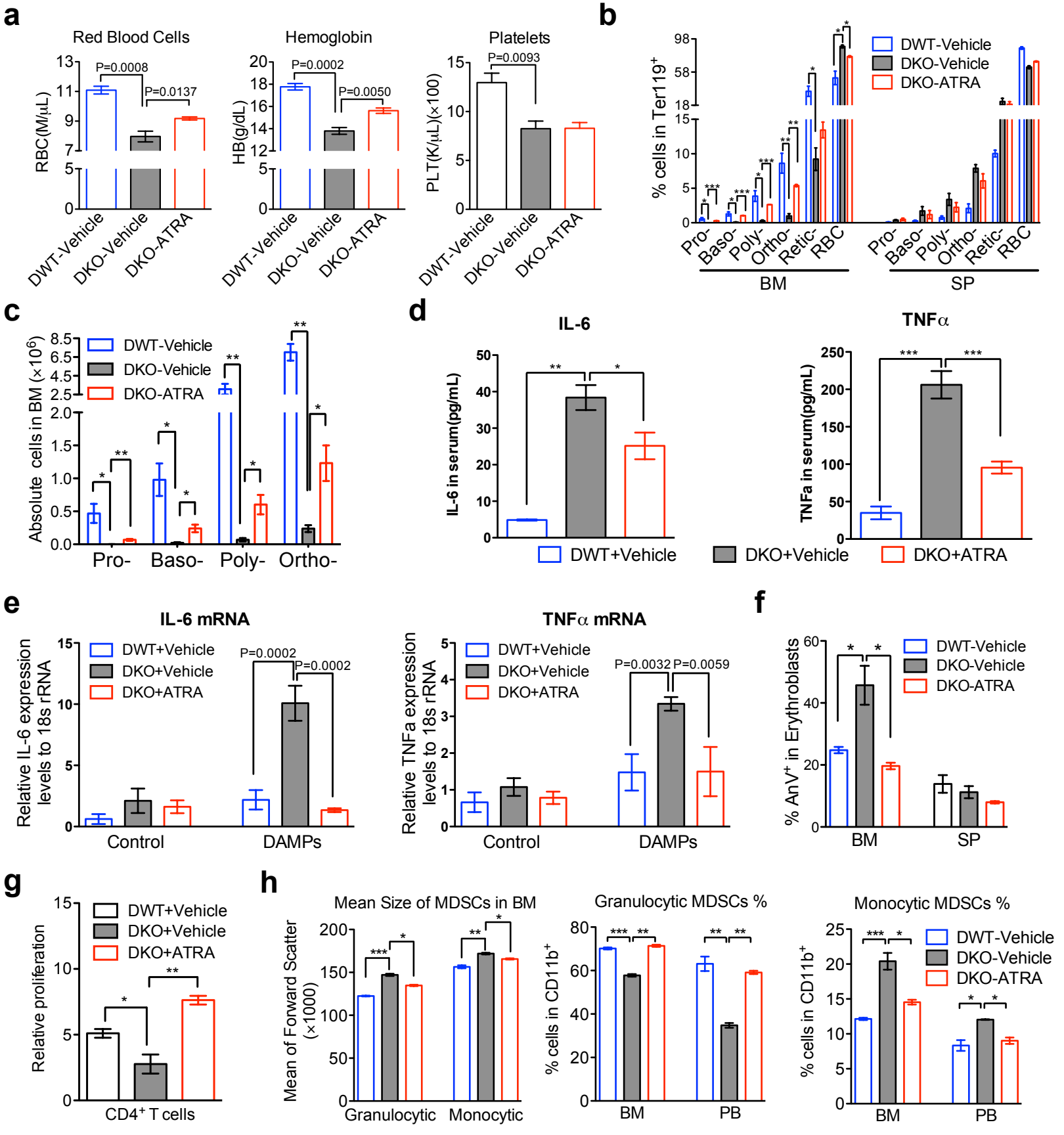



a
lethal irradiation
CD45.2+
Transplantation
Young recipient
(2-month old)
Bone Marrow from
Old (>1 year)
DWT or DKO mice
Old recipient
(> 1-year old)
b
Neutrophils
DWT
DKO
NE(K/μL)
Months
Platelets
PLT(×10³)(K/μL)
Months
c
Red Blood Cells
RBC (M/μL)
Hemoglobin
HgB (g/dL)
HCT
HCT(%)
Months
d
Red Blood Cells
RBC (M/μL)
Hemoglobin
HgB(g/dL)
HCT
HCT(%)
Months
e
Percent survival
DWT
DKO
P=0.0029
Days
f
Absolute cells in BM
(×10⁶)
DWT
DKO
P=0.0238
P=0.030
P=0.0029
P=0.014
Pro-
Baso-
Poly-
Ortho-
g
Red Blood Cells
RBC(M/μL)
Hemoglobin
HB(g/dL)
HCT
HCT(%)
Months

Mei et al. Figure 3




a
Realtive Hepcidin mRNA levels to 18s rRNA
P=0.0711,NS
DWT
DKO
b
CD71/Ter119+
DWT
DKO
Culture Time(hr)
c
CFU-E Colony Formation (% Control)
0 1 2 5
TNFα
0 1 2 5 (×10² ng/ml)
IL-6
d
%AnV+ cells in Ter119+
IL-6
TNFα
Day 1
Day 2
Day 3
e
ROShigh% cells in Ter119+
IL-6
TNFα
Day 1
Day 2
Day 3
f
Active Caspase 3+% in Ter119+
IL-6
TNFα
Day 1
Day 2
Day 3
g
% ROShigh cells in Ter119+
P=0.0236
P=0.0036
IL-6 - + + + +
NAC - -
% Active Caspase 3+ in Ter119+
P=0.0351
P=0.0003
IL-6 - + + + +
NAC - -
h
ROS MFI(×1000)
DWT
DKO
P=0.0144
P=0.0223
P=0.0011
P=0.001
P=0.0223
Ungated
Pro-
Baso-
Poly-
Ortho-
i
Mean percentage of apoptotic cells
DWT
DKO
P=0.0054
P=0.0004
P=0.0084
P=0.0094
P=0.0479
Ungated
Pro-
Baso-
Poly-
Ortho-


a
Red Blood Cells
RBC(M/μL)
P=0.0008
P=0.0137
Hemoglobin
HB(g/dL)
P=0.0002
P=0.0050
Platelets
PLT(K/μL)(×100)
P=0.0093
DWT-Vehicle
DKO-Vehicle
DKO-ATRA
b
% cells in Ter119+
DWT-Vehicle
DKO-Vehicle
DKO-ATRA
Pro-
Baso-
Poly-
Ortho-
Retic-
RBC
BM
SP
c
Absolute cells in BM (×10^6)
DWT-Vehicle
DKO-Vehicle
DKO-ATRA
Pro-
Baso-
Poly-
Ortho-
d
IL-6
IL-6 in serum(pg/mL)
TNFα
TNFa in serum(pg/mL)
DWT+Vehicle
DKO+Vehicle
DKO+ATRA
e
IL-6 mRNA
Relative IL-6 expression levels to 18s rRNA
P=0.0002
P=0.0002
TNFα mRNA
Relative TNFa expression levels to 18s rRNA
P=0.0032
P=0.0059
Control
DAMPs
f
% AnV+ in Erythroblasts
DWT-Vehicle
DKO-Vehicle
DKO-ATRA
BM
SP
g
Relative proliferation
DWT+Vehicle
DKO+Vehicle
DKO+ATRA
CD4+ T cells
h
Mean Size of MDSCs in BM
Mean of Forward Scatter (×1000)
Granulocytic
Monocytic
Granulocytic MDSCs %
% cells in CD11b+
Monocytic MDSCs %
BM
PB

Mei et al. Figure 6